Biomimetic asymmetric catalysis based on biological and synthetic macromolecular scaffolds

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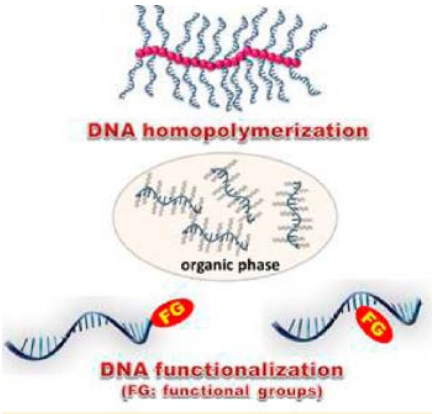
Chapter 5 Nucleic Acid Chemistry in Organic Phase: from Functionalized Oligonucleotides to DNA Side Chain Polymers

DNA-incorporating hydrophobic moieties can be synthesized by either solid-phase or solution-phase coupling. On a solid support the DNA is protected, and hydrophobic units are usually attached involving a DNA synthesizer. On the other hand, solution coupling in aqueous medium results in low yields due to the solvent incompatibility of DNA and hydrophobic compounds. Hence, the development of a general coupling method for producing amphiphilic DNA conjugates with high yield remains a major challenge. Here, we report an organic-phase coupling strategy for nucleic acid modification and polymerization by introducing a hydrophobic DNA-surfactant complex as a reactive scaffold. As a proof-of-concept, DNA-pyrene conjugates and a series of new brush-type DNA side-chain homopolymers with high DNA grafting density are produced efficiently by the transition metal-catalyzed reactions. We believe that this method is an important breakthrough in developing a generalized approach to synthesizing functional DNA molecules for self-assembly and related technological applications.



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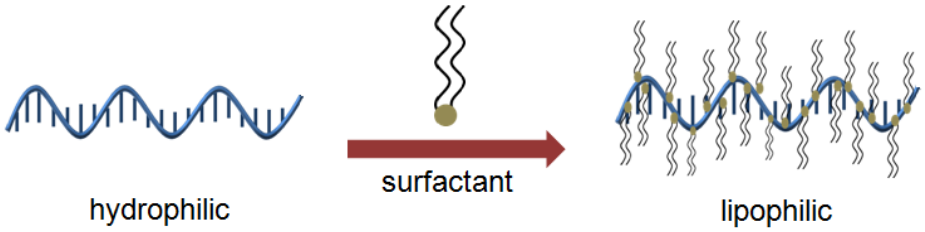
5.1 Introduction

The combination of the sequence addressability of DNA and the diversity of functional groups that may be introduced at various sites of the molecule (nucleobase, sugar, or phosphodiester backbone) by conventional synthesis[1-6] has led to the widespread implementation of DNA in diverse applications.[7-12] Conjugates of DNA with organic molecules,[13-16] polymers,[17-20] metal coordination complexes,[21] and nanoparticles,[22,23] have served as important tools in the development of new biohybrid materials and reagents that are designed for use in template-directed synthetic chemistry,[24,25] catalysis,[26,27] biomimetics,[28] magnetics,[29] (opto)electronics,[30-32] diagnostics,[33-35] biomedicine,[36-38] and therapeutics.[39,40]

Presently, solid-phase synthesis and solution-phase coupling are the two methodologies one can employ to chemically modify the natural DNA scaffold. Solid-phase synthesis of functionalized DNA most often relies on a commercially available automated DNA synthesizer. Terminal functionalization or the introduction of non-natural nucleotides can be easily integrated into the automated synthesis protocol mostly relying on phosphoramidite chemistry. Alternatively, post-synthetic modification may be carried out on the solid support outside of the synthesizer. In this case, not only phophoramidite chemistry is useful for functionalization but also other transformations are suited for DNA modification including amide formation, Michael addition, or Huisgen cycloaddition.[2,3] However, all the approaches on solid phase have the following general limitations: (I) Yields are usually lower compared to reactions in solution due to the heterogeneous character of the reaction. (II) Special care needs to be taken in solvent selection if a polymer support is employed for DNA synthesis due to solvent-dependent swelling properties of crosslinked polymer resins. (III) Finally, the new products or introduced functional groups need to be stable against the harsh basic deprotection conditions used for removing the protective groups on the nucleic acid scaffold. Thus, modification strategies of DNA based on solid-phase synthesis remain prohibitive for some well-established coupling reactions and chemical functionalities. The absence of a general methodology to introduce

various functional moieties renders DNA modification by full solid-phase synthesis a realistic approach only when significant effort can be dedicated to overcoming the many synthetic challenges involved.

On the other hand, solution-phase DNA modification in an aqueous environment has proven to be highly versatile and efficient in coupling hydrophilic molecules at various DNA positions. However, the synthesis of amphiphilic DNA hybrid materials containing hydrophobic functional moieties is less efficient due to the difficulty of finding solvents that accommodate both extreme lipophiles and hydrophilic DNA strands.[2-6] To overcome this incompatibility, we employ a method of solubilizing DNA in organic solvents by exchanging the counter-ions, which are present along the charged DNA backbone, with quaternary ammonium surfactants (Scheme 5.1). In doing so, we neutralize the charge on the DNA and provide a hydrophobic coating that can shuttle DNA into the organic phase.[41-44]



Scheme 5.1. Scheme of preparation of DNA surfactant complex.

This complexation with surfactants is not only suitable for DNA-tempaletd reactions not proceeding in water[45] but can be broadly applied as a simple, generic strategy for overcoming incompatibilities in solubility in the production of functionalized DNA molecules. As a proof-of-concept, we demonstrate that the DNA-surfactant complex permits site-specific modifications at nucleobases within a fully deprotected DNA molecule by the palladium-catalyzed Sonogashira–Hagihara coupling reaction, which was reported to be unsuccessful in the past.[46,47] Moreover, we synthesize DNA side chain homopolymers by ring-opening metathesis polymerization (ROMP), a new type of DNA polymer architecture. While statistical copolymers exhibiting DNA side chains are known,[20,33,48-50] to the best of our knowledge, such homopolymer DNA

brushes have not been realized yet.

5.2 Results and discussion

5.2.1 Site-specific functionalization of a non-standard DNA base in the organic phase

Post-synthetic modification strategies have so far mostly relied on DNA immobilized on a solid support to overcome the differences of solubility in the synthesis of amphiphilic structures. For instance, post-synthetic Sonogashira reactions on an activated nucleotide that is positioned in the middle or at the end of the sequence have been realized by treating the protected on-column oligonucleotides with a reaction mixture. However, the application of such a reaction on the same sequence while being cleaved from the solid support was reported to be unsuccessful.[46,47] To address this challenge, we have developed a strategy for the site-specific modification on nucleobases within a fully deprotected sequence by using the DNA-surfactant complex as a reaction scaffold.

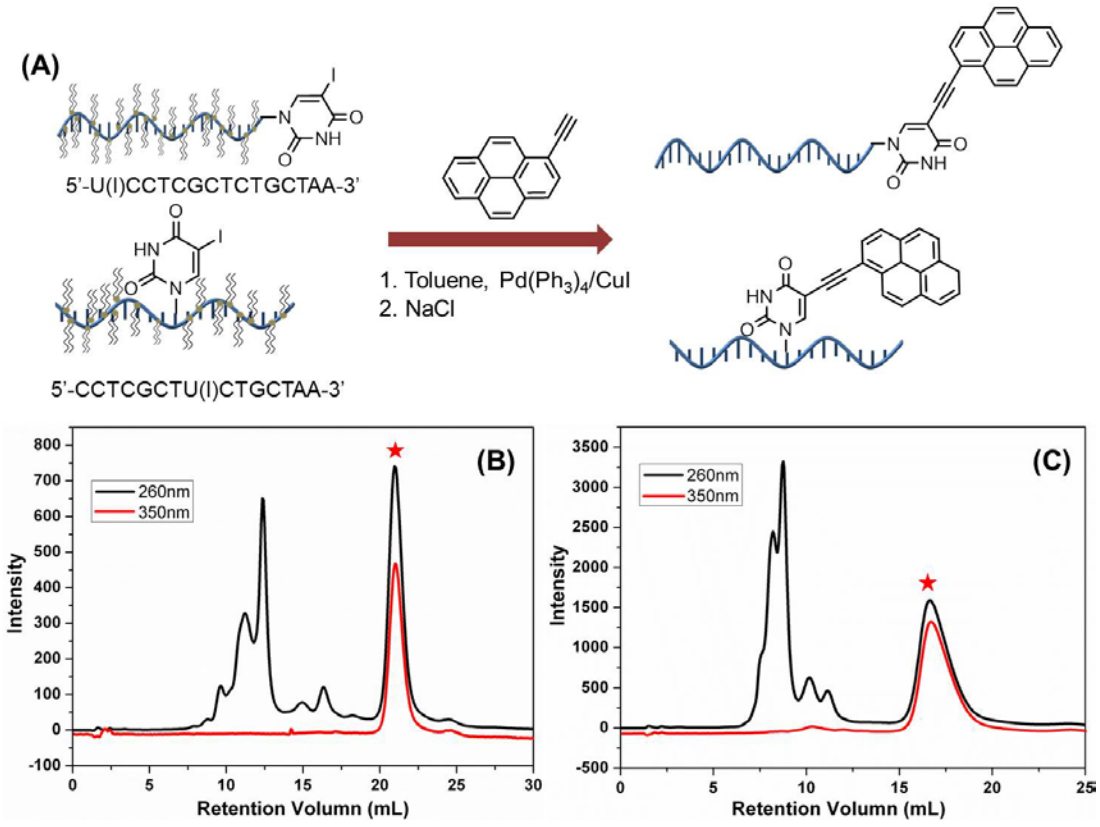


Figure 5.1. Site-specific functionalization of nucleobases by Pd-catalyzed

Sonogashira–Hagihara reaction in DMF/Et_3N. (A) Schematic of the route for the modification of 5I-dU nucleobase at terminus (U_T) or located in the middle (U_M). Reverse phase HPLC analysis of U_TDNA-PY (B) and U_MDNA-PY (C) crude products. The product elution peaks (marked with red star) where both the pyrene absorption at 350 nm and the DNA absorption (260 nm) are detected represent the DNA-PY conjugates obtained with 55% (U_TDNA-PY) and 45% (U_MDNA-PY) coupling efficiencies.

As shown in Figure 5.1, two 15-mer oligonucleotides, with the sequences U_TDNA: 5'-U(I)CCTCGCTCTGCTAA-3' and U_MDNA: 5'-CCTCGCTU(I)CTGCTAA-3', were synthesized by standard phosphoramidite chemistry. 5'-Dimethoxytrityl-5-iodo-2'-deoxyuridine 3'-[(2-cyanoethyl)-(N,Ndiisopropyl)] phosphoramidite (5I-dU phosphoramidite) was used to introduce a 5I-dU residue as the terminal and internal residue of the two oligonucleotides, which renders the oligonucleotides active for further modification by the Sonogashira–Hagihara reaction.[46] The synthesized U_TDNA and U_MDNA were confirmed by MALDI-TOF MS (Figure 5.3). However, it is necessary to point out that both of the samples contained the corresponding deiodinated side products, which could not be separated completely by HPLC. A general scheme of the pathway taken to functionalize a DNA sequence is as following: First, DNA-surfactant complexes were prepared by electrostatic complexation of oligonucleotides with the cationic surfactant didodecyldimethylammonium bromide (DDAB) in the aqueous phase. Complex formation leads to the precipitation of the DNA from the aqueous solution and allows the recovery of the surfactant-coated DNA by centrifugation. Following lyophylization, the DNA-DDAB complex is soluble in organic solvents such as DMF, DMSO, THF, toluene and $CHCl_3$. Separately, as a model coupling reagent, 1-ethynylpyrene (PY) was synthesized in accordance with methods reported in the literature.[51] Under argon atmosphere, DNA-surfactant complexes were allowed to react with PY in the presence of $Pd(P(Ph)_3)_4$ and CuI in Toluene/Et_3N at 60°C. (Figure 5.1A) The final crude products, after cation exchange with sodium chloride, were purified and analyzed by reverse phase HPLC (Figure 5.1B and 5.1C). The DNA-PY conjugates are attributed to the elution peak that simultaneously exhibits not only the DNA absorption at 260 nm but also the typical pyrene absorption at 350 nm.

Transformation efficiencies, derived from the HPLC chromatograms, are calculated to be 55% and 45% for U_TDNA-pyrene and U_MDNA-pyrene, respectively. It is noteworthy, however, that the actual coupling efficiencies would be higher if the non-reactive deiodinated U_TDNA and U_MDNA components were extracted from the starting material. The purified U_TDNA-pyrene and U_MDNA-pyrene conjugates were characterized by MALDI-TOF MS (Figure 5.4), confirming the formation of the desired products. These results showed that fully deprotected oligonucleotides containing a convertible 5I-dU nucleotide base can be effectively and precisely functionalized by Sonogashira–Hagihara reaction on the scaffold provided by the lipophilic DNA-surfactant complex.

5.2.2 Organic-phase polymerization of DNA-norbornene for side-chain DNA homopolymers

Due to the exceptional tolerance toward a variety of functional groups, ROMP provides distinct advantages over other polymerization systems for the construction of brush-type DNA/polymer hybrid materials.[33,50] Here, our novel strategy was applied for the generation of a new type of DNA brushes, i.e. DNA side chain homopolymers (Figure 5.2A). Two 5'-norbornene modified DNA macromonomers (7merNBDNA: 5'-CCTCGCT-3' and 14merNBDNA: 5'-CCTCGCTCTGCTAA-3') were synthesized and characterized by MALDI-TOF MS (Figure 5.5), after which the NBDNAs were complexed with DDAB by electrostatic interaction as described above.

We first optimized the ROMP reaction conditions for the 7merNBDNA-DDAB macromonomer. The homopolymerization was carried out using the Grubbs catalysts (first or second generation) in organic solvents (THF or $CHCl_3$). The premade monomer complex and catalyst solutions were separately purged under argon for 30 min each and then mixed together. Polymerization was allowed to proceed for 3 h at room temperature, after which it was terminated by adding ethyl vinyl ether. After cation exchange employing sodium chloride solution, the crude products were directly subjected to denaturing PAGE analysis (Figure 5.8). The reaction products exhibit

multiple distinct bands that form a ladder-like pattern, indicating the formation of DNA species with higher molecular weight. According to the optimization studies, it was found that polymerization carried out with second generation Grubbs catalyst in THF led to a crude product with the greatest number of distinct bands (Figure 5.2B). We posit that this approach has been uniquely successful where others were not because the surfactant serves the additional functions of reducing the repulsion force by shielding the negative charges and at the same time generating additional affinity between DNA complexes by hydrophobic interactions. Upon subjecting the other macromonomer (14merNBDNA) to the ROMP reaction under the optimized conditions, polymerization is also observed. However, fewer bands were observed by PAGE (Figure 5.2C), indicating a lower degree of polymerization for the bulkier monomer complex. This result is likely observed due to the steric effects that hinder the approach of norbornene units to the bulky monomer complex. As an important parameter of the polymers, the polydispersity index (PDI) of the two brushes were then calculated (see the experimental section 5.4.5 for details). It was found that both of them showed rather narrow polydispersities with a value of around 1.2, which suggests that initiation is faster than propagation. The slower propagation, again, can be explained by the steric hindrance of the macromonomers.

Subsequently, the crude DNA brushes were separated and purified by PAGE. As indicated from the analytical PAGE (Figure 5.2B), DNA brushes with different degree of polymerization showed distinct mobilities and thus they were well separated on the gel. In this respect, the one-step purification was carried out efficiently by preparative PAGE. Each band corresponds to a single side-chain DNA brush with specific polymerization degree and well defined structure (one DNA per repeating NB), indicating the monodispersity of the purified products. Taking 7merNBDNA brushes as an example, the molecular weights of the oligmers (from band 2 to band 5, Figure 5.2D) are in good agreement with the calculated values of monodisperse DNA brushes with polymerization degrees ranging from 4 to 7 (Figure 5.6). Oligmers of 14merNBDNA brushes characterized by MALDI-TOF MS also showed a constant

mass increase which is equal to the molecular weight of macromonomer (Figure 5.7). For the DNA side chain homopolymers (Figure 5.2E), the attempt of characterization by MALDI-TOF MS was unsuccessful. However, their molecular weights and polymerization degree could be reasonably estimated according to the relevant band numbers on the PAGE gel.

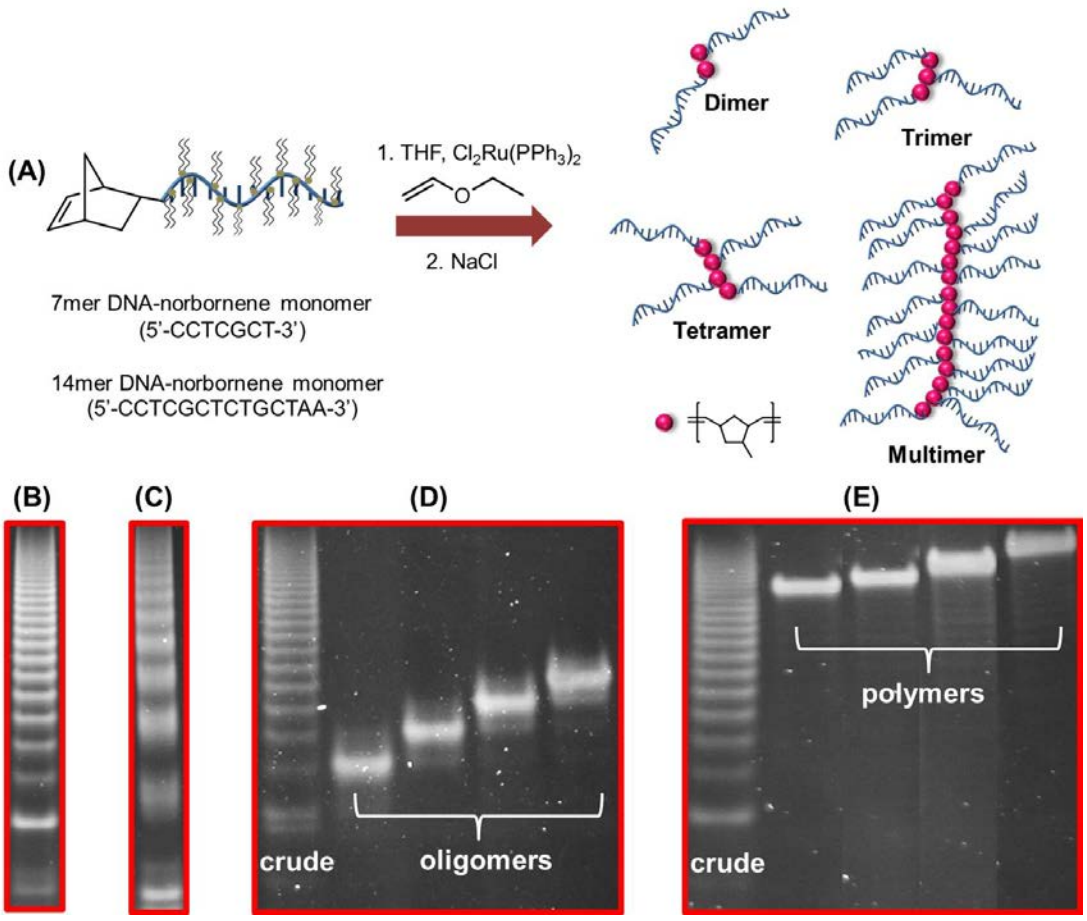


Figure 5.2. A series of DNA side-chain homopolymers obtained from DNA-norbornene (NBDNA) macromonomer. (A) Schematic of route for 7merNBDNA-DDAB and 14merNBDNA-DDAB polymerized in absolute THF by ring-opening metathesis polymerization. Denaturing polyacrylamide gel electrophoresis (PAGE) analyses of the polymerized 7merNBDNA (B) and 14merNBDNA-DDAB (C) crude products, indicate the formation of DNA brushes. PAGE purification of the 7merNBDNA brush oligomers (D) and polymers (E), where each band corresponds to a single side-chain DNA brush with specific polymerization degree.

5.3 Conclusions

In this chapter, a facile synthetic method for nucleic acid functionalization in organic

phase has been developed. Utilizing a DNA-surfactant complex as a versatile and general scaffold for DNA functionalization in the organic phase, we have incorporated hydrophobic modifications at terminal and internal DNA positions. Moreover, the polymerization of norbornene functionalized DNA-surfactant complex macromonomers allows the fabrication of novel DNA side chain homopolymers. Most importantly, the established strategy avoids the large instrumental investment of acquiring an automated DNA synthesizer, thus expanding the accessibility of problematic, custom-synthesized DNA conjugates to the level of research groups. As a consequence, this technique paves the way to the synthesis of a wide variety of amphiphilic DNA hybrids for exploring DNA applications in bio- and nanotechnology more broadly.

5.4 Experimental section

5.4.1 General

^{1}H-NMR and ^{31}P-NMR spectra were recorded on a Varian 400 (^{1}H, 400 MHz). Functional DNA molecules and amphiphilic DNA conjugates were purified by high-performance liquid chromatography (HPLC, ÄKTA DNA explorer, GE Healthcare). Analysis of the crude DNA conjugates was carried out by denaturing polyacrylamide gel electrophoresis (PAGE) (20 w% TBE gel, Invitrogen life technologies) with subsequent SYBR Gold staining, and photographs of the gels were taken with a LAS-3000 Image Reader (Fuji Photo Film (Europe) GmbH, Dusseldorf, Germany). Purification of the DNA side chain homopolymers was carried out by preparative PAGE (8 w%, Acr/Bis = 19:1, ~10 M Urea). Mass spectrometry of the synthesized DNA and DNA conjugates was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) with 3-hydroxypicolinic acid (HPA) as matrix. During all experiments, ultrapure water (18.2 MΩ) purified by a MilliQ-Millipore system (Millipore, Germany) was used.

5.4.2 Materials

Surfactant didodecyldimethylammonium bromide (DDAB) used for the DNA

complex formation was purchased from Sigma-Aldrich. 5-iodo deoxyuridine-modified DNA including UTDNA (5'-U(I)CCTCGCTCTGCTAA-3') and UMDNA (5'-CCTCGCTU(I)CTGCTAA-3'), norbornene (NB)-modified DNA including 7merNBDNA (5'-NBCCTCGCT-3') and 14merNBDNA (5'-NBCCTCGCTCTGCTAA-3') were synthesized by standard phosphoramidite chemistry.[19] Custom Primer Support C6 amino 200 was purchased from GE Healthcare Lifescience. 5'-DMT-5-iodo deoxyuridine was purchased from ChemGenes. Other solvents and reagents for DNA synthesis were aquired from Sigma-Aldrich.

5.4.3 MALDI-TOF mass spectra of the products

Functionalization of nucleotide base in organic phase

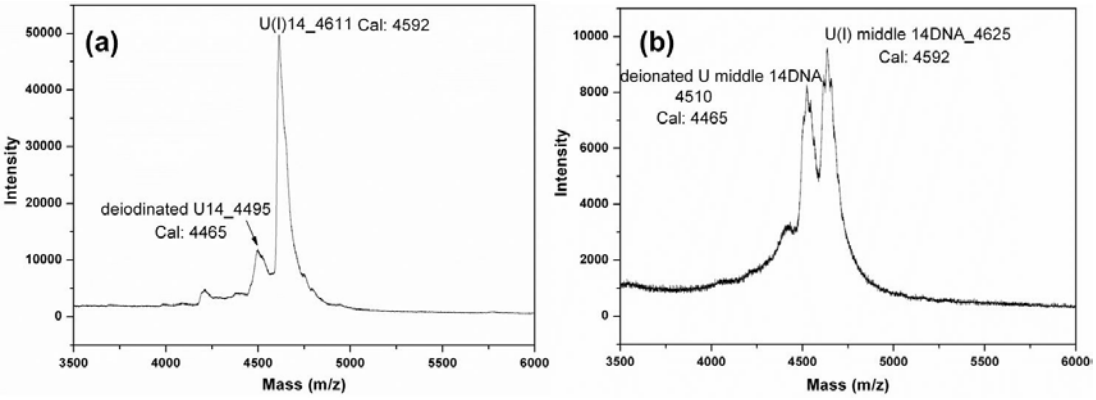


Figure 5.3. MALDI TOF mass spectra of 15mer DNA with 5I-dU at 5'-terminus (U_TDNA) and in the middle (U_MDNA) of the sequence. It is necessary to point out that deiodinated U_TDNA and U_MDNA are still present in the samples because it is difficult to separate them completely by anion exchange HPLC.

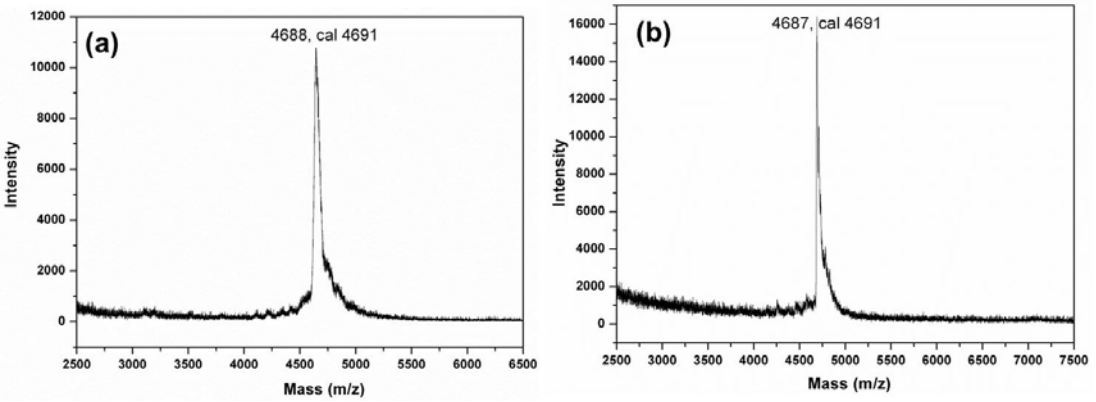


Figure 5.4. MALDI TOF mass spectra of the formed U_TDNA-PY (a) and U_MDNA-PY conjugates obtained by Sonogashira–Hagihara reaction.

Polymerization of DNA-norbornene in organic phase

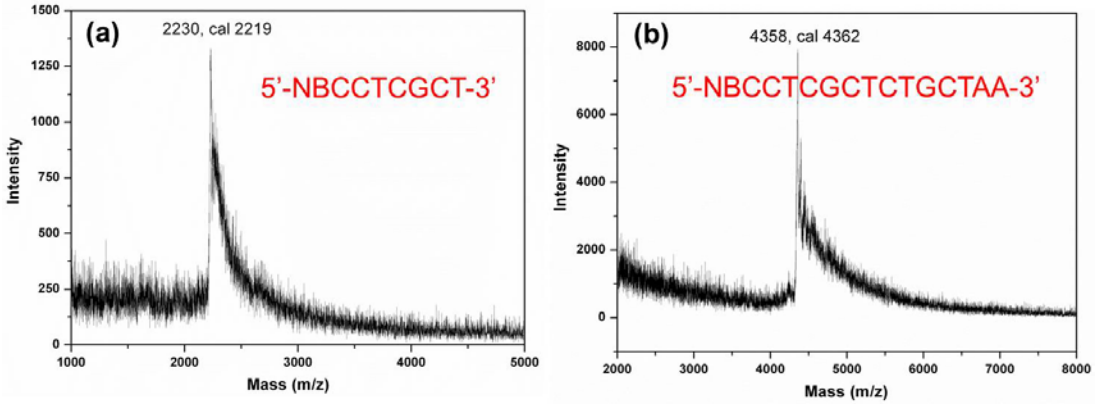


Figure 5.5. MALDI TOF mass spectra of the DNA-norbornene macromonomers 7merNBDNA and 14merNBDNA.

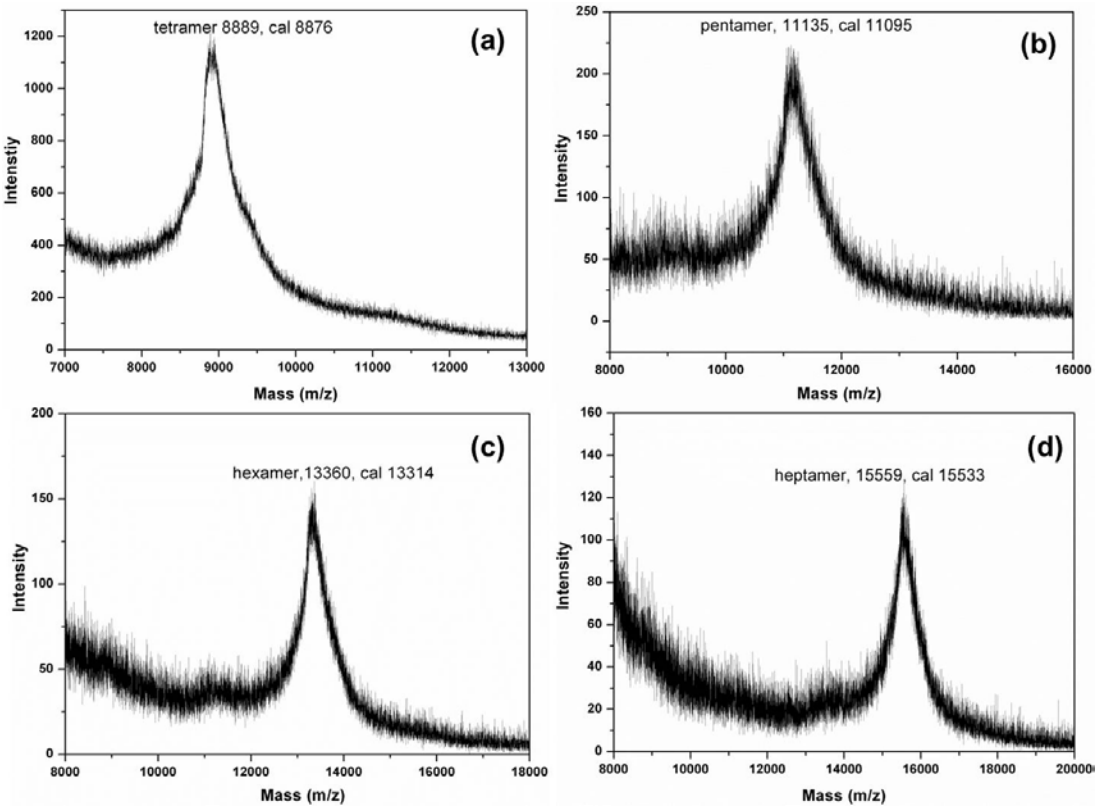


Figure 5.6. MALDI-TOF mass spectra of the oligomers (tetramer (a), pentamer (b), hexamer (c) and heptamer (d)) of polymerized 7merDNA-norbornene brushes purified by preparative PAGE.

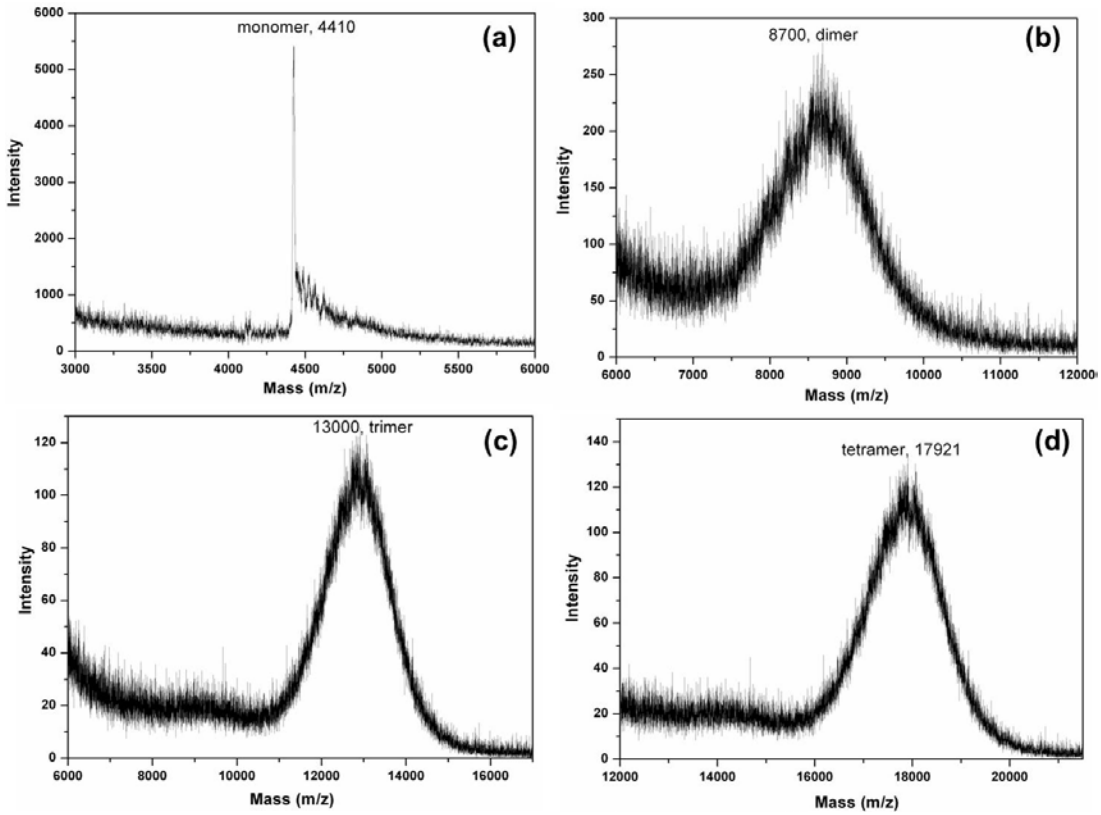


Figure 5.7. MALDI-TOF mass spectra of the oligomers (monomer (a), dimer (b), trimer (c) and tetramer (d)) of polymerized 14merDNA-norbornene brushes purified by preparative PAGE.

5.4.4 Optimization of the ROMP reactions

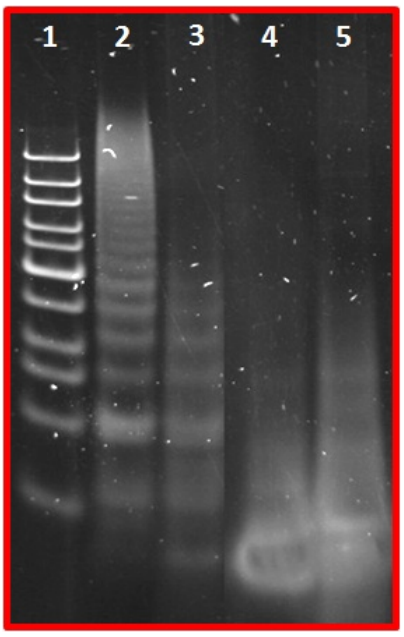


Figure 5.8. Denaturing polyacrylamide gel electrophoresis (PAGE) analysis of polymerized 7merDNA-norbornene under various conditions. 1, DNA ladder; 2, Grubss' catalyst 2nd Generation, THF; 3, Grubss' catalyst 2nd Generation, CH_3Cl; 4, Grubss' catalyst 1st

Generation, CH_3Cl; 5, Grubss' catalyst 1st Generation, THF.

5.4.5 Calculations of the PDI of brush-type DNA side chain polymers

In order to calculate the PDI, the software (ImageJ) was employed to integrate each band on the gel (**Figure** 5.2B and 5.2C) to get the relevant distribution of the resulting conjugates. From this, we then calculate the M_n and M_w of the DNA brushes according to the formulas. Finally, the PDI can be obtained from the value of M_w/M_n. The detailed calculations are as following:

Table 5.1. PDI calculation of 7mer DNA brushes

Peak	Area	N_i	M_i	$N_i * M_i$	$N_i * M_i^2$
1	5933	7%	3	0.211362	0.634087
2	2328	3%	4	0.110575	0.4423
3	3974	5%	5	0.23594	1.179699
4	5566	7%	6	0.3966	2.379598
5	6699	8%	7	0.556833	3.897831
6	6672	8%	8	0.63382	5.070563
7	6726	8%	9	0.718824	6.46942
8	6640	8%	10	0.788467	7.884672
9	6229	7%	11	0.813613	8.949741
10	5459	6%	12	0.777922	9.335063
11	5075	6%	13	0.783407	10.18429
12	4601	5%	14	0.764966	10.70952
13	4082	5%	15	0.727088	10.90633
14	3640	4%	16	0.691639	11.06623
15	2720	3%	17	0.549122	9.335082
16	2593	3%	18	0.554278	9.977011
17	1791	2%	19	0.404165	7.679126
18	2108	3%	20	0.50075	10.01501
19	1375	2%	21	0.342825	7.199327
SUM	**84213.02**	**1.00**	**SUM**	**10.56**	**133.31**
			Mn	10.56	
			Mw	12.62189	
			PDI	1.20	

Table 5.2. PDI calculation of 14mer DNA brushes

Peak	Area	N_i	M_i	$N_i * M_i$	$N_i * M_i^2$
1	8375	10%	2	0.192295	0.38459

2	16260	19%	3	0.560039	1.680116
3	14971	17%	4	0.687523	2.750091
4	13683	16%	5	0.785458	3.927289
5	10695	12%	6	0.736746	4.420475
6	8738	10%	7	0.702253	4.915774
7	5613	6%	8	0.515561	4.124485
8	4769	5%	9	0.492741	4.434669
9	3997	5%	10	0.458938	4.589382
SUM	**87100.73**	**1.00**	**SUM**	**5.13**	**31.23**
			Mn	5.13	
			Mw	6.1	
			PDI	1.19	

5.4.6 Synthesis

DNA-surfactant complex preparation.

Aqueous solutions (0.5 mM) of 5-iodo deoxyuridine modified DNA (U_TDNA and U_MDNA) and norbornene (NB) modified DNA (7merNBDNA and 14merNBDNA)) were prepared by dissolving the purified DNA in ultrapure water. In a second solution made from ultrapure water, the concentration of surfactant of didodecyldimethylammonium bromide (DDAB) was adjusted to 5-10 mM at room temperature. When the DNA and surfactants solutions (~5 mol equivalents of surfactant relative to nucleotides of the DNA) were mixed together, the insoluble complex precipitated from the aqueous phase. After centrifugation, the water and unreacted surfactants were removed, and finally, the complexes were lyophilized overnight before further dissolving in organic solvents for coupling with hydrophobic molecules.

Modification of nucleotide base by Sonogashira–Hagihara reaction.

To a round bottom flask**,** U_TDNA-DDAB (U_MDNA-CTAB) complex (0.5 μmol), pyrene-1-alkyne (10.0 μmol), [$Pd(PPh_3)_4$] (4.6 μmol) and CuI (9.0 μmol) were added. After purging with argon for 30 minutes, 2.0 ml DMF/Et_3N (v/v=1/1) was added as solvent to the flask and the reaction was allowed to proceed for 12 h at 60^{o}C. Then,

saturated NaCl aqueous solution was added to the organic mixture, which was stirred for another 3h. After desalting, the crude products were purified by reverse phase HPLC and characterized by MALDI-TOF MS.

Polymerization of DNA-norbornene by ring-opening metathesis polymerization.

The monomer complex solution (3.5 μmol in 1ml THF) and stock solution of Grubbs' catalyst (second generation, 2.8 μmol in 2ml THF) were separately purged under argon for 30 min. Polymerization was initiated by mixing 100 μL of the catalyst solution (4% mol) with the monomer solution. Polymerization was allowed to proceed for 3 h at room temperature and was terminated by the addition of ethyl vinyl ether (100 μL). After salt treatment, the crude products were directly subjected to denaturing PAGE analysis.

5.5 References

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